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Abstract: BACKGROUND Precision medicine trials targeting the epidermal growth factor receptor (EGFR) in glioblastoma patients require selection for EGFR-amplified tumors. However, there is currently no gold standard in determining the amplification status of EGFR or variant III (EGFRvIII) expression. Here, we aimed to determine which technique and which cutoffs are suitable to determine EGFR amplification status. METHODS We compared fluorescence in-situ hybridization (FISH) and real-time quantitative (RT-q)PCR data from patients screened for trial inclusion into the Intellance 2 clinical trial, with data from a panel-based next generation sequencing (NGS) platform (both DNA and RNA). RESULTS By using data from >1000 samples, we show that at least 50% of EGFR amplified nuclei should be present to define EGFR gene amplification by FISH. Gene amplification (as determined by FISH) correlates with EGFR expression levels (as determined by RT-qPCR) with receiver operating characteristics analysis showing an area under the curve of up to 0.902. EGFR expression as assessed by RT-qPCR therefore may function as a surrogate marker for EGFR amplification. Our NGS data show that EGFR copy numbers can strongly vary between tumors, with levels ranging from 2 to more than 100 copies per cell. Levels exceeding 5 gene copies can be used to define EGFR-amplification by NGS; below this level, FISH detects very few (if any) EGFR amplified nuclei and none of the samples express EGFRvIII. CONCLUSION Our data from central laboratories and diagnostic sequencing facilities, using material from patients eligible for clinical trial inclusion, help define the optimal cutoff for various techniques to determine EGFR amplification for diagnostic purposes.

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Defining *EGFR* amplification status for clinical trial inclusion

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Abstract

Background. Precision medicine trials targeting the epidermal growth factor receptor (EGFR) in glioblastoma patients require selection for *EGFR*-amplified tumors. However, there is currently no gold standard in determining the amplification status of *EGFR* or variant III (*EGFRvIII*) expression. Here, we aimed to determine which technique and which cutoffs are suitable to determine EGFR amplification status.

Methods. We compared fluorescence in-situ hybridization (FISH) and real-time quantitative (RT-q)PCR data from patients screened for trial inclusion into the Intellance 2 clinical trial, with data from a panel-based next generation sequencing (NGS) platform (both DNA and RNA).

Results. By using data from >1000 samples, we show that at least 50% of *EGFR* amplified nuclei should be present to define *EGFR* gene amplification by FISH. Gene amplification (as determined by FISH) correlates with *EGFR* expression levels (as determined by RT-qPCR) with receiver operating characteristics analysis showing an area under the curve of up to 0.902. *EGFR* expression as assessed by RT-qPCR therefore may function as a surrogate marker for *EGFR* amplification. Our NGS data show that EGFR copy numbers can strongly vary between tumors, with levels ranging from 2 to more than 100 copies per cell. Levels exceeding 5 gene copies can be used to define EGFR-amplification by NGS; below this level, FISH detects very few (if any) *EGFR* amplified nuclei and none of the samples express *EGFRvIII*.

Conclusion. Our data from central laboratories and diagnostic sequencing facilities, using material from patients eligible for clinical trial inclusion, help define the optimal cutoff for various techniques to determine *EGFR* amplification for diagnostic purposes.

Key Points

1. We show which cutoffs define EGFR amplification for diagnostic purposes.
2. Diagnostic EGFR amplification cutoffs are defined for FISH, RT-qPCR, and NGS.
3. *EGFR* gene expression can serve as a surrogate marker for *EGFR* amplification.

In glioblastomas, the most common and aggressive type of primary brain tumor, the gene encoding epidermal growth factor receptor (EGFR) is mutated in approximately half of

all tumors^{1,2} (Lassman et al, submitted). Initially, the *EGFR* locus is amplified to high copy number levels, sometimes exceeding 100 copies per tumor cell.³ Additional mutations

Importance of the Study

Precision medicine trials targeting *EGFR* in glioblastoma patients require selection for *EGFR*-amplified tumors. However, there is currently no standard to determine the amplification status of *EGFR* or *EGFRvIII* expression. In this study, we used molecular data derived from central laboratory testing and diagnostic-grade sequencing

facilities, using a clinically relevant patient population (ie, those eligible for clinical trial inclusion) to help determine which technique and which cutoffs are suitable to determine *EGFR* amplification status for diagnostic purposes. Our results are of high relevance for the selection of patients into precision medicine trials.

may evolve after gene amplification and these can include intragenic deletions, point mutations, and/or gene fusions.¹ As secondary mutations mainly arise after gene amplification, they are almost always subclonal and it is not uncommon that several of these coexist within the same tumor. Secondary mutations in *EGFR* therefore add to the intratumoral heterogeneity of glioblastomas.⁴ *EGFR* amplicons are present in cells as double minutes: extra-chromosomal and circular DNA fragments. These double minutes also contribute to tumor heterogeneity, as they are unevenly distributed across the 2 daughter cells following cell division.³

Although glioblastomas depend on *EGFR* for growth, clinical efficacy of *EGFR* tyrosine kinase inhibitors has thus far been disappointing.^{5–7} For example, in newly diagnosed glioma patients, gefitinib has no added clinical benefit when given after radiotherapy.^{8,9} Similarly, in recurrent glioma patients, erlotinib as single agent did not improve 6 months progression-free survival.^{10,11} However, novel therapies that use *EGFR* either as a target or as a marker enriched in tumors, such as vaccines, chimeric antigen receptor T cells, or antibody drug conjugates, are under development or are already in clinical trials for glioblastoma patients.^{12–16} Either way, clinical trials targeting *EGFR* require a consensus for the optimal method and cutoff to determine *EGFR* amplification status.

The randomized phase II AbbVie M14-483/EORTC 1410-BTG “Intelligence 2” trial examined whether depatux-M improves survival in *EGFR*-amplified recurrent glioblastoma patients. Trial results show a trend toward improved survival when depatux-M was given in combination with temozolomide (TMZ) compared with an alkylator only-based control arm. Longer-term follow-up confirmed improved survival (van den Bent et al, in preparation). For this trial, *EGFR* amplification was determined in a central laboratory using fluorescence in-situ hybridization (FISH), and patients harboring *EGFR*-amplified tumors were selected from >1000 screened samples. Additional molecular studies on these samples was performed which included real-time quantitative (RT-q)PCR to determine *EGFR* and variant III (*EGFRvIII*) expression and panel-based sequencing, but only of patients included in the trial (both DNA and RNA). The molecular data derived from central laboratory testing and diagnostic-grade sequencing facilities, using a clinically relevant patient population (ie, those eligible for clinical trial inclusion), help define the optimal cutoff for various techniques to determine *EGFR* amplification for diagnostic purposes. Our data can help the selection of patients into precision medicine trials.

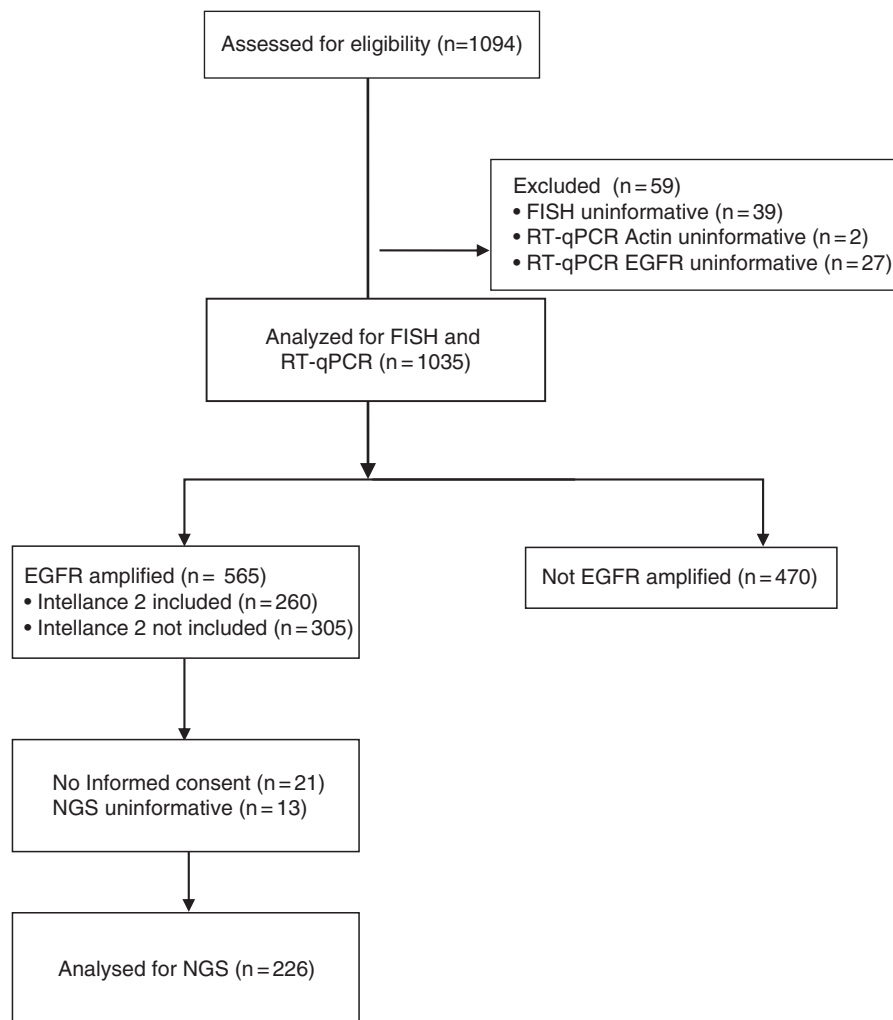
Materials and Methods

Patients

Recurrent glioblastoma patients were considered eligible for the Intelligence 2 trial (clinical trial identifier NCT02343406) if they had been diagnosed with a histologically confirmed, *EGFR*-amplified glioblastoma at first occurrence. For the screening assay, in the majority of cases (~86%), material from first surgery was used. This is possible since *EGFR* amplification is a temporally stable genetic event in the large majority of patients.^{17–19} A total of 1094 samples were screened from which 260 patients were randomized in the Intelligence 2 trial to receive either (i) TMZ or, if progressing within 16 weeks of the start of the last maintenance TMZ cycle, CCNU ($n = 26$ and $n = 60$, respectively; total $n = 86$); (ii) depatux-M alone ($n = 86$); or (iii) TMZ in combination with depatux-M ($n = 88$). A Consolidated Standards of Reporting Trials (CONSORT) diagram is shown in Figure 1. Eligibility criteria for molecular analysis were histologically confirmed de novo glioblastoma (primary) with unequivocal first progression after RT concurrent/adjuvant chemotherapy at least 3 months post radiotherapy, age ≥ 18 years, and Karnofsky performance status 60–100. All centers had to obtain approval of the study design from their local ethical boards before study activation according to national and institutional regulations. All patients gave written informed consent for trial participation and molecular testing.

Fluorescence In-Situ Hybridization

FISH was performed by one of 3 laboratories (Histogenex, Antwerp, Belgium, $n = 801$; Mosaic, Lake Forest, California, $n = 100$; and Peter MacCallum Cancer Institute, Melbourne, Australia, $n = 233$) on glioblastomas using the Vysis *EGFR* CDx Assay (Abbott Molecular; not on market) as described (Lassman et al, submitted). Reproducibility between labs for FISH and RT-qPCR was determined using a proficiency tissue panel that was run on all sites upon startup. The proficiency of laboratory personnel to perform the Abbott RealTime *EGFR* assay was evaluated during RealTime *EGFR* assay training. The training/proficiency runs consist of a panel (glioblastoma formalin-fixed paraffin embedded [FFPE] tissue sections), *EGFR* positive and negative controls processed through all steps of the *EGFR* assay procedure (RNA isolation, amplification/detection reaction setup, and operation of the m2000 RealTime System). The trainees were evaluated based on performance of



CONSORT diagram of the study population assessed for biomarkers.

assay procedure as well as results of each proficiency run. One hundred percent concordance with expected results was one of the proficiency criteria. Data from all sites was evaluated by Abbott Molecular, and results were within expected range. For FISH, 2 DNA probes labeled with spectrally distinct fluorophores hybridizing to the 7p11.2-7p12 region or the chromosome enumeration probe (CEP) 7 hybridizing to the chromosome 7 centromere were used. Slides and probe mix were denatured at 73°C for 5 minutes and then hybridized at 37°C for 14–24 hours on a ThermoBrite system (Abbott Molecular). Sample pretreatment and post-hybridization washes were performed using the Vysis Universal FFPE Tissue Pretreatment and Wash Kit (Abbott Molecular; not commercially available). Slides were reviewed using fluorescence microscopy, and FISH signal counts (copy number) for individual probes were recorded for a total of 50 nuclei. A tumor was considered *EGFR* amplified when there was focal *EGFR* gene amplification defined as an *EGFR*/CEP 7 ratio greater than or equal to 2 in $\geq 15\%$ recorded cells. Tumors with polysomy for chromosome 7 (excess copies of the entire chromosome)

but without focal amplification of the *EGFR* gene were considered to be *EGFR* nonamplified.

Real-Time Quantitative PCR

RT-qPCR was performed as described (Lassman et al, submitted). Briefly, one ≥ 5 μm^2 section containing a minimum of 50 mm² total tissue area from a FFPE tumor block was processed for RNA extraction using the Qiagen RNeasy FFPE Extraction Kit (Qiagen Sciences) according to the manufacturer's instructions. FFPE sections were deparaffinized, and proteinase K was added. The nucleic acids were de-crosslinked from formalin and DNase treated to remove DNA content. Purified RNA was combined in a 96-well plate with mastermix containing primers and probes for amplification and detection of total *EGFR* and β -actin on the Abbott m2000 RealTime System (Abbott Molecular). β -actin served as an endogenous control and to provide relative quantitative values for total *EGFR* expression in the samples. The difference (ΔCt) between β -actin cycle threshold (Ct) and total *EGFR* Ct was calculated and reported.

DNA/RNA Isolation and Sequencing

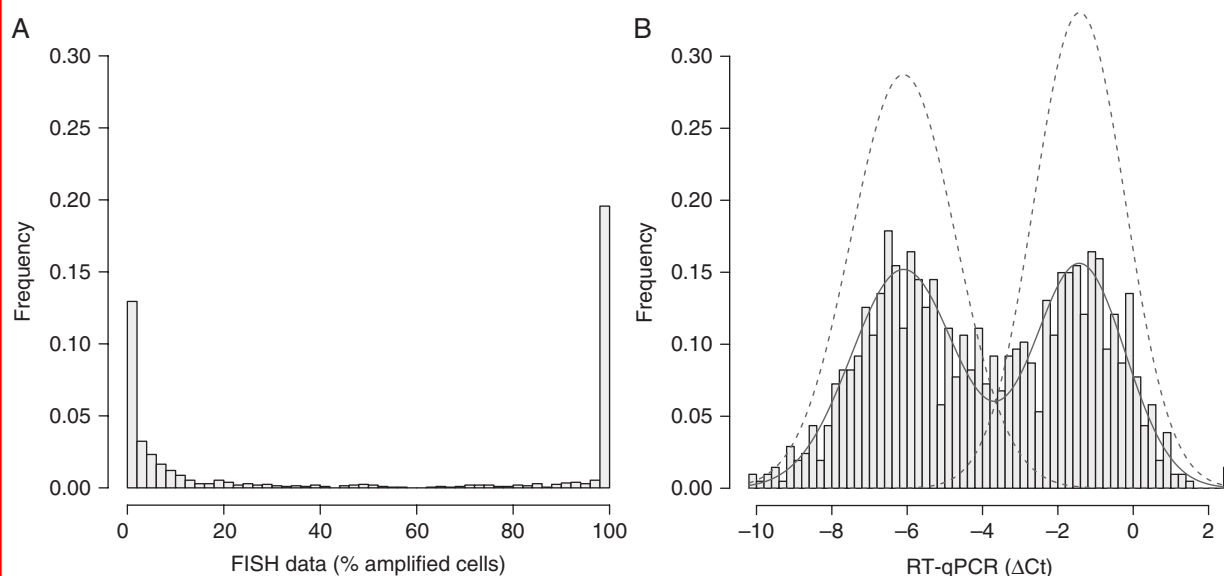
Materials, either tissue sections or tissue blocks, were centrally collected at the Erasmus Medical Center in Rotterdam. Material of 226 patients included in the Intellance 2 trial was collected. Evaluation of the area with highest tumor content was done by the pathologist (J.M.K.) on a hematoxylin and eosin stained section. Ten to twenty 5- μ m sections were then sent to Almac Diagnostics (Craigavon, UK) for macrodissection and subsequent DNA and RNA extraction using the Allprep DNA/RNA FFPE kit (Qiagen). Sequencing was done using the TruSight Tumor 170 panel (Illumina), which uses a combination of DNA and RNA sequencing to interrogate single nucleotide variations (SNVs) in ~150 genes, amplification of 59 genes, and fusion and splice variant expression in 55 genes.²⁰ Sufficient quality was obtained in 216 samples for DNA sequencing and 215 for RNA sequencing. SNV, copy number, fusion gene, and splice variant expression calling was done on the Illumina BaseSpace sequence hub using the TruSightTumor 170 App.

For each sample of the 1094 patients, one FISH assessment and one RT-qPCR value for *EGFR*, actin, and *EGFRvIII* was available. For 226 of these, the additional NGS data are available with one *EGFR* amplification level value (derived from a combination of probes on the *EGFR* locus, determined by the standard TruSight170 pipelines on the Illumina BaseSpace sequencing hub). *EGFR* amplification status by FISH was initially dichotomized as per trial inclusion (ie, using a cutoff of 15% amplified nuclei per tumor unless otherwise stated). *EGFR* amplification status as determined by RT-qPCR was dichotomized based on the bimodal distribution of the frequency histogram using Δ Ct values

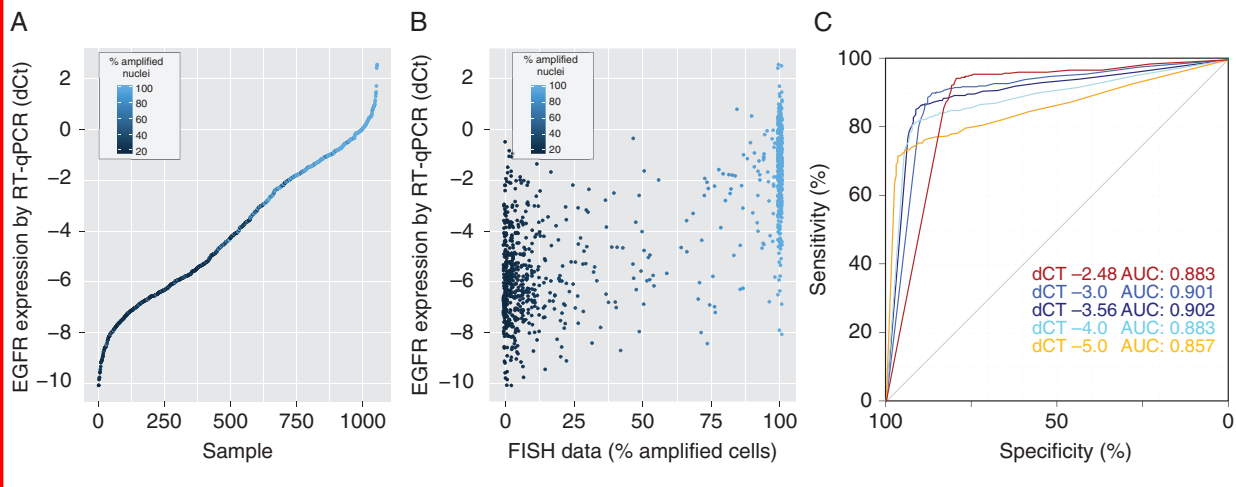
stated in the analysis. Mean and cutoff values of bimodal distributions were determined based on a finite mixture model using the Expectation-Maximization algorithm and calculated based on the “cutoff” R package.²¹ This package was also identified the cutoff point for FISH to determine *EGFR* amplification. The Δ Ct value with highest AUC (−3.56) and with a type I error rate of 0.05 (−2.48) was used to show concordance between FISH and RT-qPCR. Comparison between frequencies was done using the chi-square test. Kappa scores were calculated using the interrater reliability and agreement “irr” package in R. Where applicable, values listed with 95% confidence intervals or \pm SD.

Results

For inclusion in the Intellance 2 trial, 1094 recurrent glioblastoma patients were screened for *EGFR* amplification by FISH in a central laboratory. Of these, 1072 samples yielded informative data to determine *EGFR* amplification (a CONSORT diagram is shown in Figure 1). Fifty nuclei were counted per sample from which the percentage of *EGFR*-amplified cells was calculated. For each cell, *EGFR* amplification was defined when the ratio of *EGFR*/centromere Chr 7 was >2 . This would amount to a copy number of *EGFR* >6 per cell as most glioblastomas have gain of chromosome 7. Interestingly, and despite the fact that glioblastomas also harbor non-neoplastic stromal cells, the majority of samples either contain almost no *EGFR* amplified cells ($<15\%$ amplified nuclei) or consist entirely of *EGFR* amplified cells ($>90\%$ amplified nuclei; Figure 2).



Frequency histogram of the percentage of *EGFR*-amplified nuclei per tumor sample by FISH (A). Note that most samples either contain almost no *EGFR*-amplified nuclei or almost entirely consist of *EGFR*-amplified nuclei. The histogram of *EGFR* RT-qPCR data (B) also shows a bimodal distribution suggesting that RT-qPCR data can also be used to determine *EGFR* amplification status. Models of the 2 distributions (dashed lines) are plotted on top of the frequency histogram. The intersect of these 2 curves at −3.56 gives highest concordance between FISH and RT-qPCR data. The cutoff value for type I errors in calling *EGFR* amplification was calculated at −2.48.



RT-qPCR can function as a surrogate to determine *EGFR* amplification status. (A) Ranked ΔC_t values from RT-qPCR data show a wide range of *EGFR* expression between samples. Samples with high *EGFR* expression often had *EGFR* amplification. (B) RT-qPCR plotted against the percentage of *EGFR*-amplified cells per sample highlights the observation that higher percentages of *EGFR*-amplified cells also express *EGFR* at higher levels. (C) ROC curves show that *EGFR* expression can predict *EGFR* gene amplification by FISH. ROC curves were plotted for 4 different cutoffs (% of *EGFR*-amplified cells) to determine *EGFR* amplification by FISH.

Relatively few samples ($n = 122$, 11.6%) had intermediate numbers of *EGFR*-amplified nuclei. The FISH results therefore indicate that glioblastomas generally are not composed of mixtures of *EGFR*-amplified and non-amplified cells.

RT-qPCR was successfully performed on 1035 tumors. The histogram of ΔC_t values, plotted in Figure 2B, shows a bimodal distribution with two peaks at -6.1 ± 1.9 and -1.4 ± 1.4 ΔC_t . Ranked ΔC_t values, plotted in Figure 3A, show a wide range of *EGFR* expression between different tumors. *EGFR* expression was correlated to amplification: the samples with high expression of *EGFR* almost always contained a high number of *EGFR*-amplified cells by FISH and, conversely, samples with low *EGFR* expression contained very few (if any) *EGFR*-amplified cells (Figure 3B). A receiver operating characteristic (ROC) plot shows the strength of gene expression in determining gene amplification (Figure 3C). When *EGFR* amplification was defined as a tumor having greater than -3.56 ΔC_t *EGFR* versus actin expression value (ie, the intersect of 2 curves modeling the bimodal distribution), the area under the curve was 0.902 (95% CI: 0.881–0.922), with an optimum (ie, the point with the best sum of sensitivity and specificity) at sensitivity of 85.6% and specificity of 91.2% (95% CI: 88.1%–93.3%). At a sensitivity of 90% the specificity was 75.9% (95% CI: 60.6–88.8%). When *EGFR* amplification was defined as the optimal for type I errors (ie, 0.05), the ΔC_t *EGFR* versus actin expression value cutoff increased to -2.48 . Also at this cutoff, the area under the curve remains high, 0.883 (95% CI: 0.862–0.904), with a sensitivity of 90.1% and specificity of 83.4%. The RT-qPCR data therefore demonstrate that *EGFR* expression can function as a surrogate marker to define *EGFR* amplification. Of note, in these analyses, the optimal threshold to determine *EGFR* amplification by FISH was a percentage of *EGFR* amplified cells exceeding 77%; higher than the 15% used for clinical trial inclusion.

Concordance between FISH and RT-qPCR

		FISH Status (77%)	
		Negative	Positive
RT-qPCR status $\Delta C_t -3.56$	Negative	506	49
	Positive	69	411
		FISH Status (77%)	
		Negative	Positive
RT-qPCR status $\Delta C_t -2.48$	Negative	539	107
	Positive	36	353

The concordance between dichotomized FISH and RT-qPCR data was high: 89% using the RT-qPCR cutoff of -3.56 ΔC_t values and 86% using the cutoff optimized for type I errors of -2.48 ΔC_t values (both using a FISH cutoff of 77% amplified cells; Table 1). Of course, any increase in stringency to define *EGFR* amplification (by increasing the percentage of FISH positive cells) would reduce the number of *EGFR*-amplified tumors. Using the criteria for clinical trial inclusion (ie, >15% amplified cells), 565 of the 1035 samples tested (54.6%) were defined as being *EGFR* amplified. When increasing the stringency to define *EGFR* amplification to >77% amplified cells by FISH, this number would decrease to 460 (44.4%).

EGFRvIII expression was also determined by RT-qPCR and yielded informative data in 1049 samples. Almost all the 263 samples expressing *EGFRvIII* were also *EGFR* amplified as defined by FISH (257/263, 97.7%, $P < 0.001$; Supplementary Figure 1A), and *EGFRvIII* expressing tumors were present mainly in samples with high levels of *EGFR* expression (Figure 4, Supplementary Figure 1B),

confirming that *EGFRvIII* is expressed almost exclusively in samples with *EGFR* amplification.

EGFR Amplification by NGS

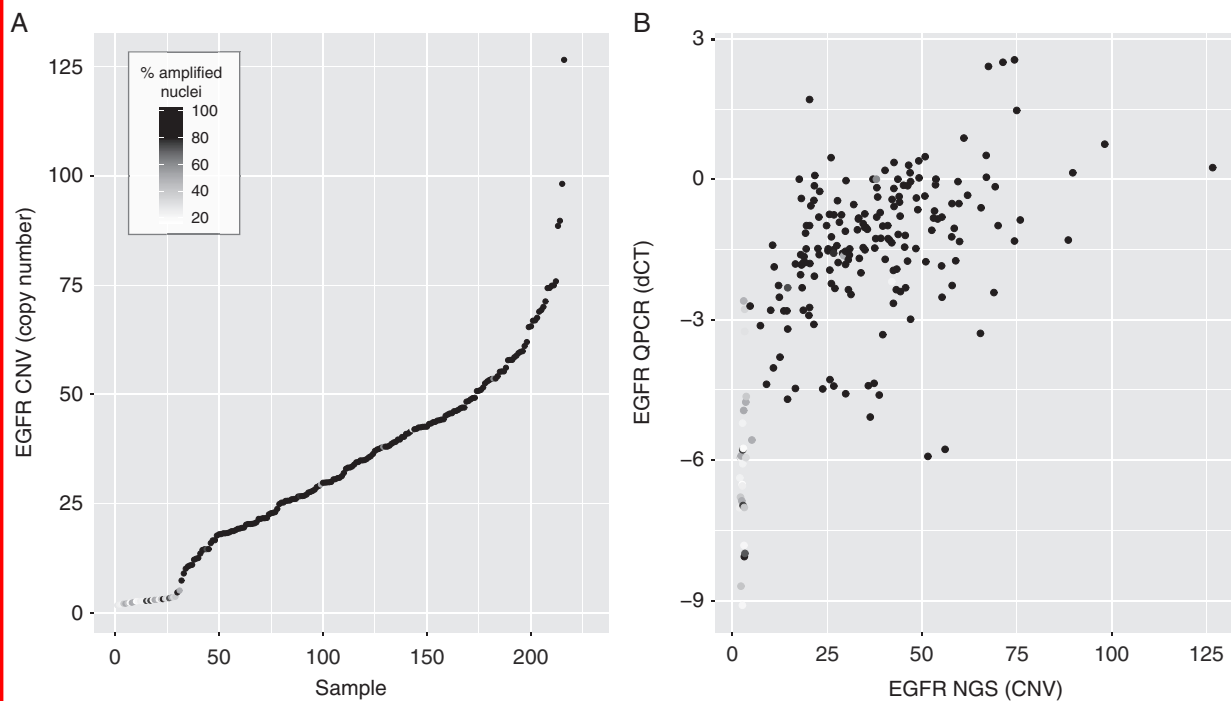
A total of 260 of the 1094 centrally screened patients were included in the Intellance 2 randomized phase II clinical trial. Patients were selected based on the presence of *EGFR* amplification by FISH only, with amplification defined as >15% of nuclei containing an *EGFR*/centromere Chr 7 ratio >2. Targeted NGS using the Illumina TruSight 170 platform was successfully performed (DNA) on 226 of these 260 samples. Ranked copy number estimates from the sequencing data are plotted in Figure 5A and confirms that most samples indeed contain high copy gene amplification. However, of the 226 samples sequenced, 30 samples (13.9%) were estimated to have fewer than 4 *EGFR* copy numbers. Most of these 30 low copy number samples also contained few *EGFR*-amplified cells as determined by FISH, and had low *EGFR* gene expression levels (Figure 5B). The observation that samples with a low percentage of amplified nuclei often show no copy number alterations by NGS suggests that FISH should use a more stringent

cutoff to molecularly determine *EGFR* amplification than the threshold used for this trial. Indeed, when *EGFR* amplification was redefined as having >50% amplified nuclei, the total number of *EGFR* amplified samples drops marginally ($n = 199/226$), but the proportion of samples containing fewer than 4 *EGFR* copy numbers by NGS was reduced 29 to 7. It should be noted, however, that cutoffs used to molecularly define *EGFR* amplification may not reflect clinical efficacy of targeted agents; precision medicine trials may use a different cutoff.

It is possible that these samples are actually *EGFR* amplified but had a low tumor cell content. However, all sections were marked for areas of high tumor content (>70% tumor cells) by a dedicated neuropathologist. Moreover, the ranked copy number variation data from NGS show a “shoulder” in *EGFR* copy number variation between <5 and >10 gene copies (Figure 5A). This shoulder can be explained by the fact that *EGFR* is present as double minutes, which facilitates rapid high copy number gains,³ and thus argues against incorrect estimation of tumor percentage (when a smoother line may be expected). Finally, none of the 29 samples express *EGFRvIII* when *EGFR* copy numbers are low (<4), whereas the percentage is markedly higher, 103 of 187 (55.1%), in samples with >4 copy



EGFRvIII expression is found in samples with high levels of *EGFR* expression. As can be seen, samples that express *EGFRvIII* (y-axis) are found in samples that have high levels of *EGFR* expression (x-axis). Dark-gray dots indicate those that have been classified as *EGFRvIII* positive tumors (ie, Ct values of *EGFRvIII* <30).



Comparison between *EGFR* amplification as determined by NGS with FISH and RT-qPCR. (A) Ranked copy number estimates by NGS show that samples with low copy numbers also had relatively few *EGFR*-amplified nuclei (darker points). Note the steep increase from <5 copy numbers to >10 copy numbers. (B) Copy number estimates by NGS correlate with gene expression derived from RT-qPCR. Samples with low copy numbers often had low levels of *EGFR* expression.

numbers ($P < 0.0001$; [Supplementary Figure 1C](#)). *EGFR* amplification precedes the rearrangement leading to *EGFRvIII* expression, and generally half of *EGFR*-amplified tumors express *EGFRvIII*.²² Therefore, the absence of *EGFRvIII*-expressing cells in samples with <5 copy numbers (and <50% FISH positive cells) also suggests that the majority of these samples are incorrectly classified, with the used FISH cutoff, as *EGFR* amplified. Our data therefore strongly suggest that the cutoff to determine *EGFR* gene amplification by FISH should be increased to a sample having >50% *EGFR*-amplified cells.

Basic patient demographics (age, sex, etc) were only collected for samples that were included in the Intellance 2 trial. Within this group, demographics between RT-qPCR-high and RT-qPCR-low cohorts were similar for patients for which NGS was attempted ([Supplementary Table 1](#)).

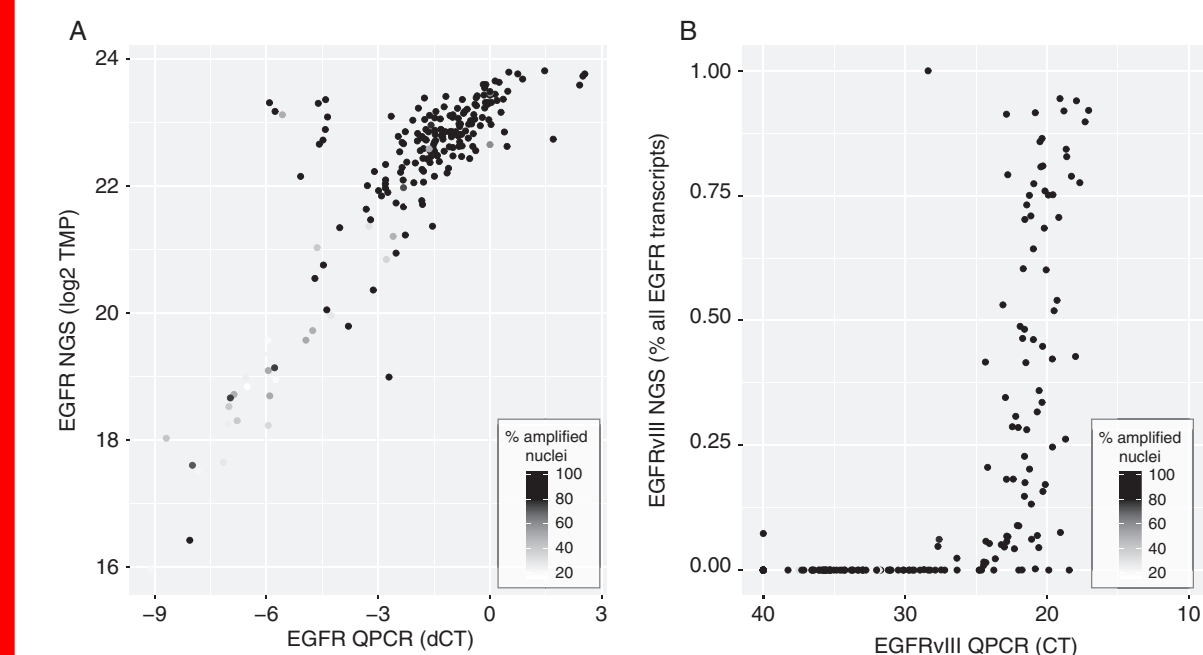
The Trusight 170 platform also includes targeted RNA sequencing of ~60 genes (including *EGFR*) and was successfully performed on 215 samples. Read depth of *EGFR* in this cohort was high; the median number of reads (combined reference and splice supporting reads) was 37 000 (interquartile range, 23 000–60 000). Comparison in *EGFR* expression between RT-qPCR and RNA-seq shows a high concordance between the 2 techniques, with most samples that have low expression on both platforms also containing relatively few *EGFR* amplified nuclei by FISH ([Figure 6A](#)). *EGFRvIII* expression was detected in 107 and 93 samples by RT-qPCR and RNA-seq respectively, with 91 samples identified by both platforms, unweighted kappa

score 0.829. The observation that RT-qPCR identifies more *EGFRvIII*-expressing samples may suggest this technique has a higher sensitivity to detect these aberrant transcripts, even despite the high read depth ([Figure 6B](#)).

Discussion

In the era of targeted treatments for cancer patients, identifying the target with an appropriate biomarker assay has become an essential part of both clinical studies and standard of care once shown effective. The biomarker assay used should be well validated: robust, reproducible, feasible, and predictive for outcome. The present study reviews 3 different assays to identify *EGFR*-amplified tumors, comparing 2 assays in the screened population and another technique for the randomized patients. In addition, the presence or absence of the *EGFRvIII* mutation is studied. *EGFRvIII* is a genomic rearrangement that occurs after *EGFR* amplification.^{22–24}

FISH for *EGFR* amplification is usually seen as a gold standard for the diagnostics of *EGFR* amplification, but what cutoffs (the % of positive nuclei, and the optimal ratio of *EGFR* vs centromere 7) should be used is unclear. For this trial, the bar to call *EGFR* amplification was set low, in view of the observation of a response in a patient with a relatively low level of *EGFR* amplification.^{13,14,25,26} An advantage of



RT-qPCR and NGS show high correlation in determining levels of *EGFR* and presence of *EGFRvIII*. (A) *EGFR* expression by RT-qPCR is highly correlated with expression derived from NGS data. (B) Samples in which *EGFRvIII* expression is identified by RT-qPCR (ie, $\Delta Ct < 30$) often also show detectable levels of *EGFRvIII* by panel-based RNA-seq.

such a low bar in a targeted treatment trial is that it allows the assessment of efficacy of the investigational compound around the borders of the assay. By using data from >1000 samples, we provide evidence that the cutoff to determine *EGFR* gene amplification by FISH should be increased, preferably to >50% of *EGFR*-amplified nuclei. Although the number of samples in the current study was large, validation of this observation in an independent cohort would strengthen our conclusions. Such cohorts are, however, at present unavailable.

Although this FISH cutoff may better determine *EGFR* amplification for diagnostic purposes, response prediction in precision medicine trials (ie, the predictive power of *EGFR* amplification) may use different cutoff points. For example, when *EGFR*-amplified cells exert a dominant effect on the non-amplified neoplastic cells (whereby the non-amplified neoplastic cells depend on the *EGFR*-amplified cells), targeting the minority of *EGFR*-amplified cells may already provide clinical benefit. The observation of patients with relatively low levels of *EGFR* amplification respond to the combination of depatux-M + TMZ supports this notion.

Our data also demonstrate that *EGFR* expression can serve as a surrogate marker to determine *EGFR* gene amplification. However, where *EGFR*-FISH has a bimodal distribution, *EGFR* expression is much more a continuum. This continuum makes it more difficult to define the optimal cutoff for *EGFR* amplification. Of note, the bimodal distribution observed by FISH demonstrates that most tumors either have no amplified cells or consist entirely of

amplified cells. This indicates that the frequency of mosaic amplification of various oncogenes as previously reported is therefore likely relatively low.²⁷

In summary, the data described in the current study, obtained from central laboratories and diagnostic sequencing facilities and using material from patients eligible for clinical trial inclusion, help define the optimal cutoff for various techniques to determine *EGFR* amplification for selection into precision medicine trials. In the end, the most optimal cutoff should be established based on evidence of clinical activity in the positive versus the negative biomarker population. That analysis is currently ongoing. Regardless, although our study shows that these tests correlate, there is a clear gray zone in which samples test positive in one assay but not in another assay.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

amplification | biomarker | *EGFR* | *EGFRvIII* | FISH | glioblastoma | screening

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Authorship statement. Conceptualization, PJF, MvdB. Methodology, PJF. Investigation, IdH, JMK. Writing, original draft, P.J.F. Writing, review, and editing, all authors. Funding acquisition, PJF. Resources, ME, J-MS, AW, J-SF, EF, PMC, MW, PA, JL, EB. Data curation: MM, TG. Supervision, PJF, MvdB.

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